

Applicant : Katia Georgopoulos
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Attorney's Docket No.: 10287-067001

REMARKS

Applicant hereby submits that the enclosures fulfill the requirements under 37 C.F.R. §1.821-1.825. The amendments in the specification merely insert the paper copy of the Sequence Listing and sequence identifiers in the specification. No new matter has been added.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment.

Please apply any charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 8/6/01


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"Version With Markings to Show Changes Made"

In the specification:

Paragraph beginning at page 7, line 30, has been amended as follows:

The Ikaros gene is active in the early differentiation of lymphocytes, e.g. T cells and B cells. The gene encodes a family of unique zinc finger proteins, the Ikaros proteins. The proteins of the Ikaros family are isoforms which arise from differential splicing of Ikaros gene transcripts. The isoforms of the Ikaros family generally include a common 3' exon (Ikaros exon E7, which includes amino acid residues 283-518 of the mouse Ikaros protein represented by SEQ ID NO:40, and amino acid residues 229-461 of the human Ikaros protein represented by SEQ ID NO:[2 38] but differ in the 5' region. The Ikaros family includes all naturally occurring splicing variants which arise from transcription and processing of the Ikaros gene. Five such isoforms are described in copending U.S. patent application 08/121,438, filed September 14, 1993. The Ikaros family also includes other isoforms, including those generated by mutagenesis and/or by *in vitro* exon shuffling. The naturally occurring Ikaros proteins can bind and activate (to differing extents) the enhancer of the CD3δ gene, and are expressed primarily if not solely in T cells in the adult. The expression pattern of this transcription factor during embryonic development show that Ikaros proteins play a role as a genetic switch regulating entry into the T cell lineage. The Ikaros gene is also expressed in the proximal corpus striatum during early embryogenesis in mice.

Paragraph beginning at page 11, line 24, has been amended as follows:

Fig. 1 is a map of the DNA sequence of a murine Ikaros cDNA and the desired amino acid sequence encoded thereby (SEQ ID NO:1 and 37, respectively).

Paragraph beginning at page 11, line 26, has been amended as follows:

Fig. 2 is a partial sequence of a human Ikaros cDNA and predicted amino acid sequence (SEQ ID NO:2 and 38, respectively).

Paragraph beginning at page 11, line 27, has been amended as follows:

Fig. 3 is a depiction of the partial amino acid composition of the IK-1 cDNA, including Ex3, Ex4, Ex5, Ex6, and Ex7 (SEQ ID NO:40).

Paragraph beginning at page 44, line 21, has been amended as follows:

As used herein, the term "exon", refers to those gene (e.g. DNA) sequences which are transcribed and processed to form mature messenger RNA (mRNA) encoding an Ikaros protein, or portion thereof, e.g. Ikaros coding sequences, and which, at the chromosomal level, are interrupted by intron sequences. Exemplary exons of the subject Ikaros proteins and genes include: with reference to SEQ ID NO:40 (mIk-1), the nucleotide sequence encoding exon 1/2 (E1/2) corresponding to Met-1 through Met-53; the nucleotide sequence encoding exon 3 (E3) corresponding to Ala-54 through Thr-140; the nucleotide sequence encoding exon 4(E4) corresponding to Gly-141 through Ser-196; the nucleotide sequence encoding exon 5 (E5) corresponding to Val-197 through Pro-237; the nucleotide sequence encoding exon 6 (6) corresponding to Val-238 through Leu-282; the nucleotide sequence encoding exon 7 (E7) corresponding to Gly-283 through Ser-518; with reference to SEQ ID NO:[2] 38 (hlk-1), the nucleotide sequence encoding exon 3 (E3) corresponding to Asn-1 through Thr-85; the nucleotide sequence encoding exon 4 (E4) corresponding to Gly-86 through Ser-141; the nucleotide sequence encoding exon 5 (E5) corresponding to Val-142 through Pro-183; the nucleotide sequence encoding exon 6 (6) corresponding to Val-184 through Leu-228; the nucleotide sequence encoding exon 7 (E7) corresponding to Gly-229 through Ser-461. The term "intron" refers to a DNA sequence present in a given Ikaros gene which is not translated into protein and is generally found between exons. The term "gene" refers to a region of chromosomal DNA which contains DNA sequences encoding an Ikaros protein, including both exon and intron sequences. A "recombinant gene" refers to nucleic acid encoding an Ikaros protein and comprising Ikaros exon sequence, though it may optionally include intron sequences which are either derived from a chromosomal Ikaros gene or from an unrelated chromosomal gene. An exemplary recombinant gene is a nucleic acids having a sequence represented by any of SEQ ID NOS:1-7 or 13.

Paragraph beginning at page 49, line 30, has been amended as follows:

The Ikaros protein shown in Fig. 1 (mIk-2) is comprised of 431 amino acids with five CX₂CX₁₂HX₃H (SEQ ID NO:36) zinc finger motifs organized in two separate clusters. (See also Fig. 4.) The first cluster of three fingers is located 59 amino acids from the initiating methionine, while the second cluster is found at the C terminus of the protein 245 amino acids downstream from the first. Two of the finger modules of this protein deviate from the consensus amino acid composition of the Cys-His family of zinc fingers; finger 3 in the first cluster and finger 5 at the C terminus have four amino acids between the histidine residues. This arrangement of zinc fingers in two widely separated regions is reminiscent of that of the Drosophila segmentation gap gene Hunchback. Similarity searches in the protein database revealed a 43% identity between the second finger cluster of Ikaros and Hunchback at the C terminus of these molecules. This similarity at the C terminus of these proteins and the similar arrangement of their finger domains raises the possibility that these proteins are evolutionary related and belong to a subfamily of zinc finger proteins conserved across species.

Paragraph beginning at page 78, line 4, has been amended as follows:

The primer extension protocol used is according to Ausubel et al. (1999) *Cell Immunol.* 193(1):99-107 (Primer Extension) with a few modifications. Briefly, total RNA was prepared from Thymus, Spleen and Liver tissue using the guanidinium method (Ausubel et al. (1999)) (Single-Step RNA Isolation from Cultured Cells or Tissues). Subsequently poly (A)⁺ RNA was isolated using the Oligotex procedure (Qiagen). The protocol is described in "Oligotex mRNA Handbook" Qiagen Inc. 1995. 1x10⁵ cpm of a kinased and gel purified oligo was precipitated with 7.5 ug poly(A)⁺, 20 µg glycogen, 0.3M NaAc, pH 5.5 in 100 µl final volume through the addition of 270 µl of 100% ethanol. The pellet was washed with 100%ethanol and then air-dried. Subsequently, the pellet was resuspended in 30 µl 1x hybridization (150mM KCl; 10 mM Tris-Cl, pH 8.3; 1 mM EDTA), incubated at 85°C for 10 minutes and then transferred to a 30°C waterbath for 12 hours. The hybridization solution was brought to a final volume of 200 µl with H₂O, then precipitated with 400 µl ethanol. The pellet was washed with 70% ethanol, air dried and resuspended in 18.4 µl 1x reverse transcription buffer (4 µl of 5x first strand buffer

(GibcoBRL); 0.4 µl of 0.1 M DTT; 8 µl of 2.5mM dNTPs (Boehringer); 6 µl of H₂O), 0.6 µl of PRIME RNase inhibitor (5'AΣ3', Inc.) and 1 µl of reverse transcriptase (Superscript II, Rnase H Reverse Transcriptase, GibcoBRL) was added. This was incubated in a 42° waterbath for 2 hours. Subsequently, 1µl of Ribonuclease H (GibcoBRL) was added and incubated for 30 minutes at 37°C. The solution was then Phenol/Cloroform/isoamylalcohol (50/49/1) extracted after the addition of 150 µl STE. Then the DNA was precipitated with 500 µl ethanol. After a washing (70% ethanol) and air drying, the pellet was resuspended in 10 µl loading buffer (80%(vol/vol) formamide; 1 mM EDTA pH 8.0; 0.1% Bromophenol Blue; 0.1% Xylene Cyanol). Before loading on a 6% acrylamide/bisacrylamide (29:l), 7 M urea gel the samples were incubated at 80°C for 5 minutes. As a size reference a sequencing reaction was run next to the sample. Figure 9B shows the autoradiography of a characteristic primer extension analysis done with a P32 labeled primer that lies in exon 2 (C29). C29 primer sequence: cct tca tct gga gtg tca ctg act g (SEQ ID NO:[__] 27).

Paragraph beginning at page 78, line 31, has been amended as follows:

For RACE analysis, primer C29 was hybridized to 7.5ug poly (A)+selected RNA and reverse transcribed as described in '5'RACE System for Rapid Amplification of cDNA Ends' kit from GibcoBRL (Cat. No. 18374-025). The resulting cDNA was 3'tailed with dCTP using the terminal deoxynucleotide transferase (GiccoBRL). The product was then PCR amplified with the nested primer C50 and a poly G /adaptor primer (GibcoBRL). As a negative control for the PCR reaction, the product of the PCR reaction was used with the exception that it lacked the 3'poly C tail (no TdT reaction). C50 primer sequence: ctg aaa ctt ggg aca tgt ctt g (SEQ ID NO:[__] 28).

Paragraph beginning at page 81, line 22, has been amended as follows:

5'Ex1BH1 primer sequence (non hybridizing sequence underlined): aaa gga tcc gaa cat aac tat gga tca gcc (SEQ ID NO:[__] 29).

Paragraph beginning at page 81, line 24, has been amended as follows:

3'ExAgeI primer sequence (no hybridizing sequence underlined): ttt acc ggt gtc ttc agg
tta tct cct gc (SEQ ID NO:[] 30).

Paragraph beginning at page 82, line 15, has been amended as follows:

Transgenic mice were made through an oocyte injection protocol as described (find reference). The mice were bred and maintained under sterile conditions in a pathogen-free animal facility at Massachusetts General Hospital. Mice were 4-8 weeks of age at the time of analysis. The mice were genotyped for GFP by PCR analysis using the following primer combination: GFPup3: cgt aaa egg cca caa gtt ca (SEQ ID NO:31) GFPdown3: ctt gaqa gtt cac ctt gat gc (SEQ ID NO:32). Cycling conditions were: 95°C 5 min, 80°C add Taq, (94°C 45 sec., 58°C 45 set, 72°C 45 sec.) x 28, 72°C 10 min., 4°C until taken out.

Paragraph beginning at page 84, line 4, has been amended as follows:

Although Ikaros is normally expressed in B cells and neutrophils, it is also expressed at its highest levels in differentiating thymocytes and mature T cells. Georgopoulos (1997) *Curr. Opin. Immunology* 9(2):222-227. Thus, additional regulatory elements must work in concert with the Ikaros promoter regions to direct expression in the T lineage. To determine the regulatory region(s) responsible for the Ikaros-T cell specific activity, the transcriptional potential of one of the most prominent DNaseI HSS present in the Ikaros locus in both the thymus and spleen was tested. A 4.7 kb EcoRI fragment containing two out of the three (T1/TS2) DNaseI HSS sites present in the ϵ cluster was introduced at the 3'end of the R10-GFP reporter (Figure 10B, R10-GFP-11). Briefly, the construct for transgenic line R10-GFP-11 was generated as follows. The R10-GFP construct was modified so that it no longer contained a KpnI site at the 5' of the gene. Additionally, a KpnI site was introduced between the SacII and SacI sites at the 3' of the construct. This resulted in construct R10-GFP-11. A 1oxP site containing vector was generated by cloning a 1oxP site between SalI and HindII and another 1oxP site between BamHI and XbaI of Bluescript II KS. For that, two annealed oligonucleotide were generated that contained a SalI cohesive end and a HindII cohesive end flanking a 1oxP site

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(see sequences 5'top and 5' bottom). Similarly, two other oligonucleotides were generated and annealed that contained a BamHI and an XbaI site flanking the loxP sequence (see sequences 3'top and 3'bottom). This resulted in vector BS-loxP. DNase I HS ε T1/TS2 was subcloned as a 4.6 kb EcoRI fragment into BS-loxP in 3'to 5'orientation. This resulted in construct BS-loxP-11. Subsequently, BS-loxP-11 was digested with SacII and KpnI and cloned in an equally digested R10-GFP-mK. This resulted in construct R10-GFP-11. The insert was released from the vector backbone in a Sall digest and prepared for microinjection. 5'top sequence: tcg acg atc gat cga tcg atc act tcg tat aat gta tgc tat acg aag tta tta agc tt (SEQ ID NO:[] 33). 5'bottom sequence: gat cca taa ctt cgt ata atg tat gct ata cga agt tat tt (SEQ ID NO:[] 34). 3'top sequence: gat cca taa ctt cgt ata atg tat gct ata cga agt tat tt (SEQ ID NO:[] 34). 3'bottom sequence: cta gaa ata act tcg tat agc ata cat tat acg aag tta tgg atc c (SEQ ID NO:[] 35).